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Glucose-6-phosphate dehydrogenase is required for extracellular polysaccharide production, cell motility and the full virulence of *Xanthomonas oryzae* pv. *oryzicola*

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ABSTRACT

Glucose-6-phosphate dehydrogenase (Zwf) catalyzes conversion of glucose 6-phosphate into gluconate 6-phosphate for Entner–Doudoroff (ED) and pentose phosphate pathways in living organisms. However, it is unclear whether the Zwf-coding gene is involved in pathogenesis of phytopathogenic bacterium. In this report, we found that deletion mutation in *zwf* of *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*), led the pathogen unable to effectively utilize glucose, sucrose, fructose, mannose and galactose for growth. The transcript level of *zwf* was strongly induced by glucose, sucrose, fructose, mannose and galactose than that by the NY medium (non sugar). The deletion mutagenesis in *zwf* also altered the transcript level of key genes, such as *rpfF*, *rpfG* and *clp*, in diffusible signal factor (DSF)-signaling network. In addition, the deletion mutation in *zwf* impaired bacterial virulence and growth capability in rice leaves, reduced bacterial cell motility and extracellular polysaccharide (EPS) production. The lost properties mentioned above in the *zwf* deletion mutant were completely restored to the wild-type level by the presence of *zwf in trans*. All these results suggest that *zwf* is required for the full virulence of *Xoc* in rice leaves by involving carbohydrate metabolisms that impact bacterial DSF-signaling network.

1. Introduction

Xanthomonas oryzae pv. *oryzicola* (*Xoc*), the yellow-pigmented gram-negative bacterium, is the causal agent of bacterial leaf streak (BLS) in rice (*Oryza sativa*). The pathogen enters rice leaves through stomata or wound, propagates in the substomatal cavity and then colonizes the parenchyma apoplast to cause BLS in rice leaves [1–3]. BLS is prevalent and of increasing importance throughout Asia, especially in China [4]. In the past, substantial progress has been made to elucidate molecular mechanisms of the *Xoc*-rice pathosystem [1–7]. One important aspect of the *Xoc*-rice pathosystem is the ability of bacterium to obtain nutrients from host rice to establish successful infection [6,7]. However, little is known about the links between bacterial nutrients acquisition and pathogenesis in this field.

It is well known that cell–cell communication, often referred to as quorum sensing (QS), is commonly observed in bacterium. The diffusible signal factor (DSF)-dependent QS system is a relatively new regulatory mechanism and is likely conserved in *Xanthomonas* spp. [8,9]. In DSF-signaling network, RpfF is responsible for the synthesis of DSF signal molecule. RpfC (sensor kinase) and RpfG (response regulator protein) constitute a two-component system essential for sensing and transducing the DSF signal [6,8]. Subsequently, the global regulator Clp (cAMP-regulatory protein) recognizes the DSF signal, and then regulates diverse biological functions including EPS production, cell motility, biofilm dispersal [6,8]. The acetyl-CoA, which roots in carbohydrate metabolism, is the carbon precursor for the synthesis of DSF signal molecule. However, little is known about whether carbohydrate metabolism genes are involved in the synthesis of DSF signal molecule, or in altering the transcript level of key genes in DSF-signaling network.

To our knowledge, the complete genome sequence data show that several strains of *X. oryzae*, such as KACC10331 [10], MAFF311018 [11], PXO99^{A} [12] and BLS256 [13], possess genes encoding all of the enzymes involved in the glycolysis, Entner–Doudoroff (ED), gluconeogenesis, pentose phosphate pathways and tricarboxylic acid (TCA) cycle. *X. oryzae* mainly employ ED, pentose phosphate pathways and terminal oxidation mediated

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by the TCA cycle to break down carbohydrates for energy and carbon molecules [6,14]. It has been proved that carbohydrate metabolism is central for bacterial extracellular polysaccharide (EPS) production and the full virulence in host plants [6,14–18]. ED is the main pathway implicated in carbon metabolisms and the synthesis of EPS [15,16]. In plant tissue, the major carbohydrates are as follows: sucrose, glucose, fructose and C4-dicarboxylic acids [19,20]. Glucose and sucrose utilization has been shown to be the best carbon sources for bacterial EPS production [16,17], and are required for the full virulence in *Xanthomonas* spp. [14,18].

Currently, several genes, like *ppsA* [20], *aceA* [20], *mls* [20], *glk* [16], *gapA* [14], *kgtP* [7], and *fbaB* [6], which involved in carbohydrate metabolism of *Xanthomonas* spp. have been investigated in some details. Previous studies showed that they are involved in utilization of various carbon sources, EPS production and the full virulence of pathogen, but not in DSF-signaling network. However, little is known about genes involving bacterial carbohydrate metabolism process. For example, glucose-6-phosphate dehydrogenase (Zwf), which converts glucose 6-phosphate into gluconate 6-phosphate, plays a crucial role in ED and pentose phosphate pathways. In this report, we investigated whether the Zwf-coding gene involved in pathogenesis of *Xoc* in rice leaves, and how Zwf played roles in carbohydrates acquisition, EPS production, cell motility and DSF-signaling network.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The plasmids and strains used in this study are listed in Table 1. *Escherichia coli* strains were routinely grown in Luria–Bertani (LB) medium (5 g L^{-1} yeast extract, 10 g L^{-1} NaCl, 10 g L^{-1} tryptone, 15 g L^{-1} agar) at 37 °C [21]. All *Xoc* strains were grown at 28 °C in NB (1 g L^{-1} yeast extract, 3 g L^{-1} beef extract, 5 g L^{-1} poly-peptone, 10 g L^{-1} sucrose), NA (NB with 15 g L^{-1} agar), NAN (NA without sucrose), NAS (NA with 100 g L^{-1} sucrose), NY (NB without beef extract and sucrose), the non-carbohydrate minimal (NCM) medium (2 g L^{-1} $(NH_4)_2SO_4$, 4 g L^{-1} K_2HPO_4, 6 g L^{-1} KH_2PO_4, 0.2 g L^{-1} $MgSO_4 \cdot 7H_2O$) [6], or rice suspension cells [22] when required. Antibiotics were added at the following concentrations when required: kanamycin (Kan), 25 μg ml^{-1}; rifampicin (Rif), 50 μg ml^{-1}; ampicillin (Amp), 100 μg ml^{-1}; spectinomycin (Sp), 50 μg ml^{-1}; and streptomycin (Sm), 50 μg ml^{-1}.

Table 1
List of bacterial strains and plasmids used in this study.

Strains or plasmids	Relevant characteristics[a]	Reference or source
E. coli		
DH5α	F⁻ Φ80d*lacZ* ΔM15Δ(*lacZYA-argF*)U169 *endA1 deoR recA1 hsdR17*(r_K^- m_K^+) *phoA supE44* λ⁻ *thi-l gyrA96 relA1*	Clontech, Palo Alto, CA, U.S.A.
***X. oryzae* pv. oryzicola**		
RS105	The wild-type, Chinese race 2, the causal agent of bacterial leaf streak in rice; Rif^r	Lab collection [1,3–6,25]
RΔ*hrpG*	A *hrpG* deletion mutant of strain RS105; Rif^r	[45]
RΔ*hrpX*	A *hrpX* deletion mutant of strain RS105; Rif^r	[45]
RΔ*hrcV*	A *hrcV* deletion mutant of strain RS105; Rif^r	[6]
RΔzwf	A *zwf* deletion mutant of strain RS105; Rif^r	This study
CRΔ*zwf*	RΔ*zwf* harboring pCzwf; Rifr, Spr, Sm^r	This study
Plasmids		
pMD18-T	pUC *ori*, cloning vector; Amp^r	Takara
pKMS1	6.4 kb, Suicide vector derivative from pK18mobGII, $sacB^+$; Kan^r	Lab collection [4,25]
pHM1	Broad-host range cos parA IncW derivative of pRI40; Spr, Sm^r	Lab collection [4,25]
pKΔzwf	A 911 bp fusion fragment cloned in pKMS1 for a 1172 bp deletion in *zwf*; Kan^r	This study
pCzwf	pHM1 expressing *zwf* under its own promoter; Sp^r	This study

[a] Amp^r = ampicillin resistance, Kan^r = kanamycin resistance, Rif^r = rifampicin resistance, Sp^r = spectinomycin, Sm^r = streptomycin resistance.

2.2. DNA manipulation

DNA manipulations were performed as described previously [23]. Mobilization of plasmids into *Xoc* was performed as described by Turner [24]. Kits for isolating genomic and plasmid DNA and for purifying DNA from agarose gels were purchased from Axygen (Beijing, China). Restriction enzymes and DNA ligases were used according to the manufacturer's instructions (Takara, Dalian, China). Oligonucleotide primers for sequencing or PCR reaction were purchased from Jinsite Biotechnol. Co. (http://www.Jinsite.com) and listed in Table S1. PCR was performed with *Ex*-Taq (Takara Bio Inc., Dalian, China). DNA sequences were analyzed with the VECTOR NTI software package (Infomax; Invitrogen, Shanghai, China).

2.3. Construction and complementation of the non-polar deletion mutation in zwf

An in-frame deletion mutation of *zwf* was constructed in *Xoc* RS105 strain using homologous recombination and pKMS1 as a suicide vector [25]. The genome sequence of *Xoc* BLS256 strain as the reference [13], two flanking fragments, left (302 bp) and right (609 bp) to *zwf* (Fig. S1), were amplified using the genomic DNA of RS105 strain as the template and the primers *zwf*-1F/*zwf*-1R and *zwf*-2F/*zwf*-2R (Table S1), respectively, and then cloned into pMD18-T vectors (Takara, Dalian, China), respectively. After confirmed by sequencing, the two fragments were digested and cloned into the vector pKMS1 at *Sma*I and *Xba*I sites, resulting in a recombinant plasmid pKΔzwf (Table 1). The plasmid pKΔzwf (50 ng μl^{-1}) was electroporated into competent cells of the RS105 strain recipient and plated for single-colony selection on NAN plates with kan (25 μg ml^{-1}) [4,25]. Colonies from the initial homologous crossover event which grew on NAN/kan (25 μg ml^{-1}) were transferred to NBN broth, grown for 12 h at 28 °C, and plated on NAS medium. Sucrose-resistant colonies were replica streaked onto NA and NA plus kan (25 μg ml^{-1}) plates, respectively [4,25]. Deletion mutations were confirmed by PCR amplification with pair primers (1F/2R and 3F/3R). One (*RΔzwf*) of the confirmed deletion colonies (Table 1) was used for further study.

In order to complement *RΔzwf*, a 1791 bp DNA fragment containing the entire *zwf* (from 360 bp upstream of the start codon to the stop codon) was amplified by PCR using the total genomic DNA of *Xoc* RS105 strain as the template and the primer pair *zwf*-F/*zwf*-R (Table S1). After confirmed by sequencing, the amplified DNA fragment was cloned into pHM1 vector at *Hin*dIII and *Kpn*I sites to create a recombinant plasmid pCzwf (Table 1). Plasmid pCzwf was transferred into *RΔzwf* by electroporation. The transconjugants carrying pCzwf were screened on NA plates with Sp or Sm (50 μg ml^{-1}), and one representative complementation strain (CRΔzwf) was verified by colony PCR and used for further study (Table 1).

2.4. Rice suspension cells culture

Oryza sativa ssp. *indica* cv. Shanyou63, susceptible to *Xoc* RS105 strain, was used for callus induction. Seeds were dehulled and sterilized in 70% ethanol for 10 min, transferred to a solution containing 50% commercial bleach with a few drops of Tween-20 for 30 min, and then soaked in 1% $HgCl_2$ for 15 min. Sterilized seeds were washed 5 times with sterile distilled water and incubated on N6 medium containing 2, 4-D (5 mg L^{-1}) at 28 °C in darkness. Actively growing calli were then selected and transferred to liquid N6 medium containing 5 mg L^{-1} 2, 4-D and 1 mg L^{-1} kinetin (KT).

Rice calli were incubated in the dark and transferred by sub-culturing at 7-day intervals at a dilution of 1:5 (inoculum: fresh medium). Generally, large amounts of rice suspension cells were obtained after 4–5 weeks of sub-culturing; at this time, single round rice cells could be observed under the microscope.

2.5. Hypersensitive response (HR) and pathogenicity assays

HR and pathogenicity assays were performed as described previously [3,4]. *Xoc* strains were assessed for their ability to cause disease symptoms and multiply in rice seedling leaves (cv. Shanyou63; two-weeks old) by infiltration with needleless syringes and in adult rice leaves (cv. Shanyou63; two-months old) by the leaf needling method, with bacterial suspensions adjusted to 3×10^8 CFU/ml ($OD_{600} \approx 0.6$). The *Xoc* strains were also tested for the ability to elicit an HR on tobacco leaves (cv. benthamiana) by infiltration with needleless syringes, with bacterial suspensions adjusted to 3×10^8 CFU/ml ($OD_{600} \approx 0.6$). Plant phenotypes were scored for HR in tobacco leaves 24 h post-inoculation, for water soaking in rice seedling leaves 3 days post-inoculation, and after 14 days post-inoculation for lesion length in adult rice leaves. All plants were grown in growth chambers at 25 °C with a 12-h photoperiod. Every experiment was repeated at least three times.

2.6. Measurement of bacterial growth capability in rice leaves and in NCM medium supplemented with different carbohydrates

The fresh *Xanthomonas* bacterial suspensions at 3×10^8 CFU/ml ($OD_{600} \approx 0.6$) were infiltrated into the intercellular spaces of fully expanded leaves of rice seedling by infiltration with needleless syringes at three spots on each leaf. Three 0.8 cm diameter leaf discs were harvested with a cork borer from each infiltration area after infiltration. After being sterilized in 70% ethanol and 30% hypochlorite, the leaf discs were homogenized in 1 ml of sterilized distilled water. Diluted homogenates were plated on NA plates with appropriate antibiotics. Bacterial CFU were counted after incubation at 28 °C for 3–4 days. Experiments were repeated at least three times.

As to the detection of bacterial growth influenced by different carbohydrates, the fresh *Xanthomonas* bacterial cells were collected and washed twice with sterilized distilled water, and re-suspended to $OD_{600} \approx 0.05$ in 20 ml of NCM medium supplemented with 0.5% (w/v) different carbohydrates (glucose, fructose, sucrose, mannose, galactose or pyruvate). For each time point, 200 μl of each culture was removed and determined by measuring OD_{600} against the medium blank. Data presented are from a representative experiment; the experiment was repeated independently at least three times.

2.7. Real-time quantitative RT-PCR assays

The transcript level of tested genes was assayed by real-time quantitative RT-PCR with corresponding primer pairs (Table S1). The fresh *Xanthomonas* bacterial cells were collected and washed twice with sterilized distilled water, and re-suspended to $OD_{600} \approx 2.0$ by sterilized distilled water. Then, the bacterial suspension was inoculated into rice suspension cells or various mediums, incubated at 28 °C for 16 h. Total RNAs were extracted using the Trizol reagent (Takara, Dalian, China) according to the manufacturer's protocol, and digested with RNase-free DNase I (TaKaRa, Dalian, China) to remove potential traces of genomic DNAs. cDNA synthesis was conducted with AMV random primers provided by the manufacturer (Takara, Dalian, China). Real-time quantitative RT-PCR was performed on the Applied Bio-systems 7500 real-time PCR System using SYBR *Premix ExTaq*™ (Takara, Dalian, China) with the *16S rRNA* as the internal standard. The comparative-threshold method was used to calculate the relative mRNA level with respect to the corresponding transcript level in the wild-type. All real-time quantitative RT-PCR were performed in two independent biological experiments with three replicates in each test.

2.8. Cell motility assay

To test cell motility, the fresh bacterial cells were collected and washed twice with sterilized distilled water, and then re-suspended to $OD_{600} \approx 0.3$ by sterilized distilled water. Then, 2 μl of bacterial suspension was spotted onto NY medium (non sugar) plate and NY medium plate supplemented with 0.5% (w/v) glucose containing 0.3% agarose, and then incubated at 28 °C for 2 days. The diameter of the area occupied by bacterial cells was measured, and the values were used to indicate the cell motility of bacterium. The experiment was repeated three times.

2.9. Measurement of EPS production

To estimate EPS production, the fresh, equivalent *Xanthomonas* bacterial cells were grown in 100 ml of NB and NY medium alone,

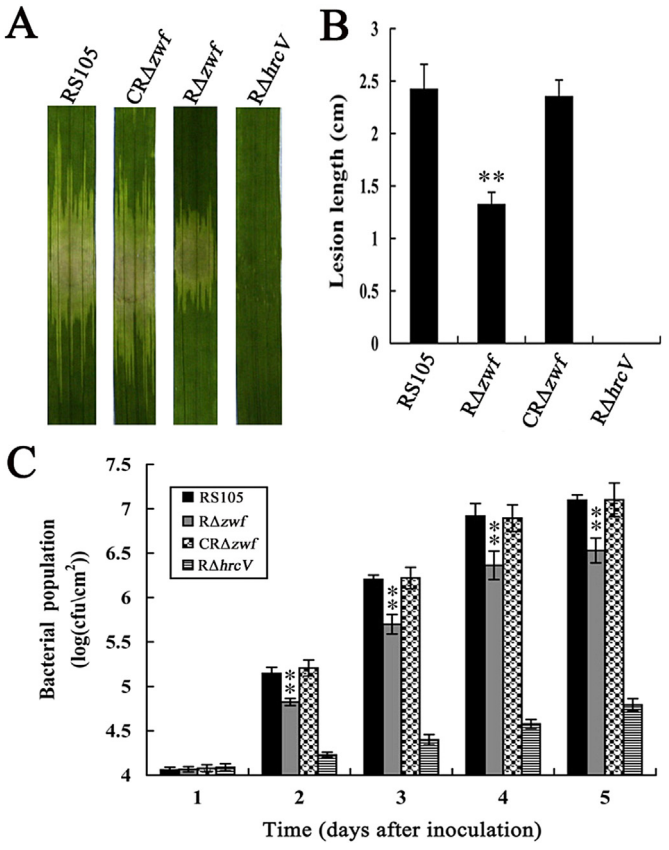

Fig. 1. The pathogenicity, virulence and growth capability tests in host rice leaves by the *zwf* deletion mutant of *Xoc*. (A) Water soaking symptoms caused by different *Xoc* strains adjusted to 3×10^8 CFU/ml ($OD_{600} \approx 0.6$) in rice seedling leaves (cv. Shanyou63; two-weeks old) by infiltration with needleless syringe. Photographs were taken 3 days after inoculation. (B) Lesion length was measured 14 days after inoculation into adult rice leaves (cv. Shanyou63; two-months old) by the leaf-needling method. Values are the means ± standard deviations (SD) from three repeats, each with five leaves. (C) Growth capability of bacteria in inoculated leaves of rice seedling (cv. Shanyou63; two-weeks old). Bacteria were recovered from the inoculated leaves every day for a period of 4 days post-inoculation, and homogenized in sterilized distilled water. The homogenates were diluted and plated on NA plates with appropriate antibiotics. Bacterial CFU were counted after incubation at 28 °C for 3–4 days. Data are means ± SD from three replicates. The different symbol in each horizontal data column results from a paired, two-tailed Student *t* test relative to the wild-type. $^{**}P = 0.01$; $^{*}P = 0.05$.

and NY medium supplemented with 2% (w/v) various sugars (glucose, fructose, sucrose, mannose, galactose or pyruvate) at 28 °C with constant shaking at 200 rpm for 5 days. EPS was precipitated from the culture supernatant with ethanol, and dried to constant weight at 55 °C, and weighed as previously described by Tang et al. [26]. Every experiment was repeated at least three times.

3. Results

3.1. *zwf* is required for the full virulence and growth capability of *Xoc* in rice leaves

In order to investigate the biological function of *zwf*, we constructed a non-polar *zwf* deletion mutant (named *RΔzwf*; Table 1) by homologous suicide plasmid integration (Fig. S1) (see Materials and Methods for details). Simultaneously, a complemented strain, designated as CRΔzwf (Table 1), was constructed by transferring the recombinant plasmid pCzwf (Table 1), which carries the entire *zwf*, into *RΔzwf*.

The pathogenicity and virulence assay for *Xoc* strains were performed in rice leaves by needleless syringe and the leaf-needling methods [4], respectively. 3 days after inoculation, the *zwf* deletion mutant showed remarkable reduced water soaking symptoms in rice seedling leaves relative to the wild-type, while CRΔzwf and the wild-type produced similar disease symptom (Fig. 1A). 14 days after inoculation, *RΔzwf* showed a mean lesion length of 1.35 cm in adult rice leaves, which is half less than a mean lesion length of 2.45 cm caused by the wild-type and CRΔzwf. Statistic analysis showed that the mean lesion length caused by RΔzwf was significantly shorter than that caused by the wild-type and CRΔzwf ($P = 0.01$) (Fig. 1B), indicating that *zwf* is required for the full virulence of *Xoc*.

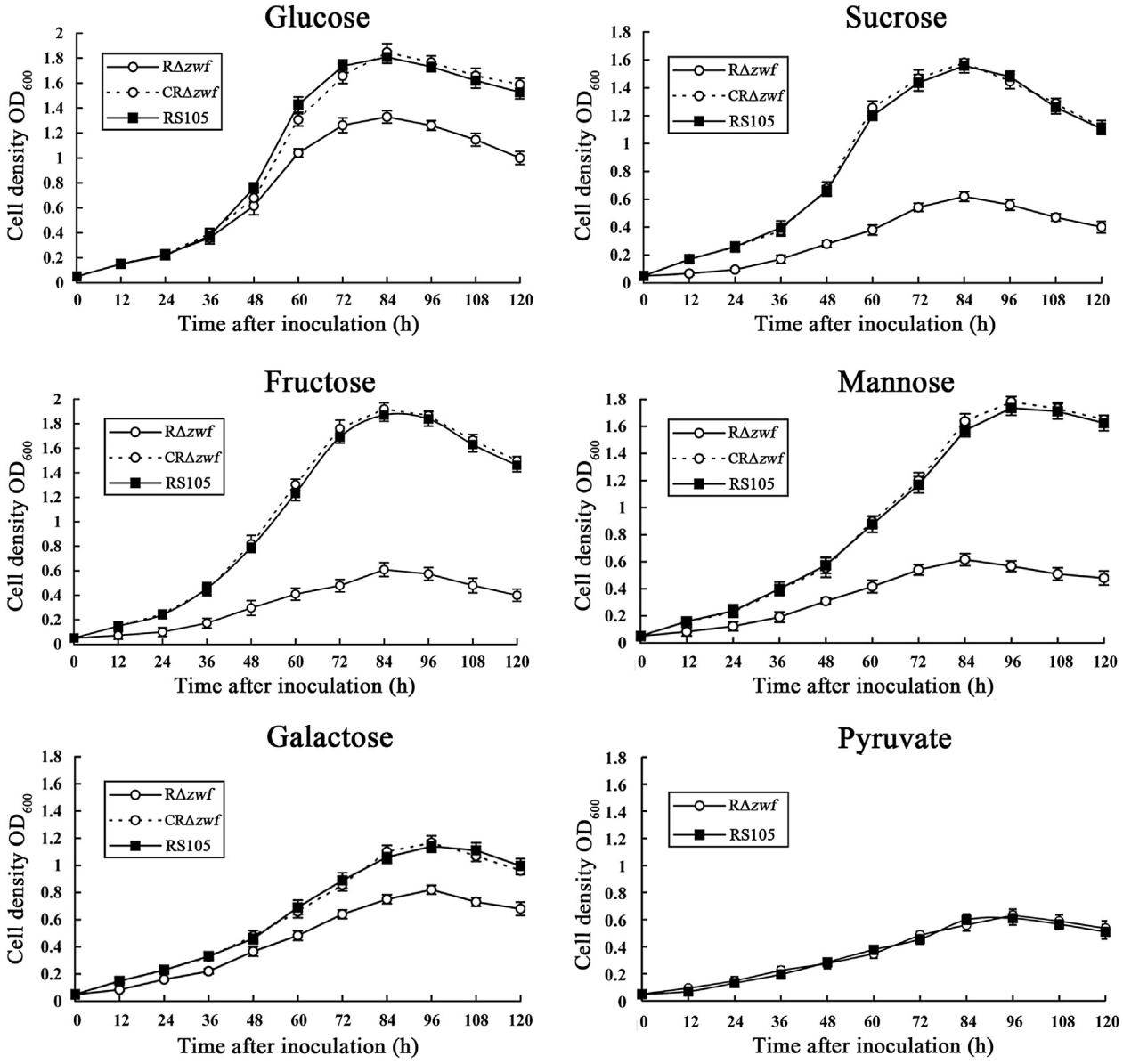

Fig. 2. Growth capability of *Xoc* in sole carbon media. *RΔzwf*, the *zwf* deletion mutant; CRΔ*zwf*, the complemented strain of *RΔzwf* with the entire *zwf*; RS105, the wild-type. The initial concentration of the tested strains was adjusted to $OD_{600} \approx 0.05$ with NCM medium supplemented with glucose, sucrose, fructose, mannose, galactose or pyruvate as the sole carbon source. Aliquots were taken in triplicate at intervals of 120 h after incubation at 28 °C, and bacterial growth was determined by measuring OD_{600} against the medium blank. Values given are means ± SD from three replicates.

To determine whether deletion mutation in *zwf* results in a decrease in the proliferation of *Xoc* in rice leaves, the bacterial cells from the infected rice leaves were recovered and calculated as CFU. As shown in Fig. 1C, in the infected rice leaves, the number of the bacterial cells with deletion mutant was significantly lower than that of the wild-type at each of the test points. The growth capability of *RΔzwf* in rice leaves could be completely restored by *zwf in trans*, whereas the type III secretion system (T3SS) deletion mutant (RΔ*hrcV*) did not grow as well as it in inoculated rice leaves. Taken together, these results indicated that *zwf* affects *Xoc* growth capability in rice leaves.

3.2. zwf is important for Xoc to utilize various carbohydrates

In *Xanthomonas* spp., ED, in conjunction with TCA, has been confirmed to be the predominant pathway for glucose catabolism, and only 8–16% of glucose is routed into pentose phosphate pathway [6,27]. *RΔzwf* grew identically as the wild-type in a non-sugar NY medium, indicating that *RΔzwf* was not auxotrophic. To further examine the effect of the Zwf on carbohydrate utilization, the growth capability of *Xoc* strains grown in NCM medium supplemented with glucose, sucrose, fructose, mannose or galactose as the sole carbon source was tested. We found that growth capability of the *zwf* deletion mutant was evidently slower than that of the wild-type when glucose, sucrose, fructose, mannose or galactose was used as the sole carbon source (Fig. 2), suggesting that the capability of the *zwf* deletion mutant to utilize these sugars is diminished. However, the *zwf* deletion mutant grew normally as the wild-type in NCM medium supplemented with pyruvate as the sole carbon source. The growth capability of CRΔzwf was identical to that of the wild-type in NCM medium supplemented with different carbohydrates (glucose, sucrose, fructose, mannose or galactose) as the sole carbon source (Fig. 2), indicating that the capability of the *zwf* deletion mutant to utilize various carbohydrates could be restored completely by *zwf in trans*. These results collectively indicated that *zwf* is important for *Xoc* to utilize carbohydrates of glycolytic carbon sources, but not required for utilization of the gluconeogenic ones.

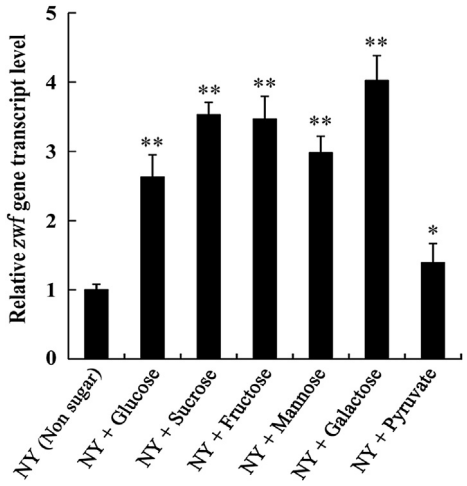

Fig. 3. Expression analysis of *zwf* in *Xoc* by real-time quantitative RT-PCR. RNAs were isolated from cultures of the wild-type grown in NY medium alone and NY medium supplemented with 0.5% various carbohydrates (glucose, sucrose, fructose, mannose, galactose or pyruvate) for 16 h. The relative mRNAs levels of *zwf* were calculated with respect to the level of the corresponding transcript in NY medium alone, with the *16S rRNA* as the internal standard. Data are means ± SD from three replicates. The different symbol in each horizontal data column results from a paired, two-tailed Student *t* test relative to the wild-type. $^{**}P = 0.01$; $^{*}P = 0.05$.

3.3. Various carbohydrates have different effects on the transcript level of zwf in Xoc

It was reported that carbohydrates present in plants may serve as inducers or inhibitors of virulence-associated gene expression in *Xanthomonas* spp. [6,28–30]. In order to determine whether the different carbohydrates have some effect on the transcript level of *zwf* in *Xoc*, we employed a real-time PCR to investigate the transcript level of *zwf* after the wild-type grew for 16 h in NY medium alone and in NY medium complemented with 0.5% glucose, sucrose, fructose, mannose, galactose or pyruvate, respectively. We found that the tested five sole carbons remarkably induced the transcript level of *zwf*, excepting that pyruvate had unconspicuous effect on the transcript level of *zwf* (Fig. 3). The results above demonstrate that the transcript level of *zwf* may be enhanced by hexoses involving in the conversion of glucose 6-phosphate into gluconate 6-phosphate.

3.4. Deletion mutation in zwf negatively affects cell motility and EPS production of Xoc

To investigate whether the deletion mutation in *zwf* results in the change of cell motility, we investigated the cell motility of *RΔzwf*. On the NY tested plate, there was no significant difference ($P = 0.05$) in cell motility among all of the strains (*RΔzwf*, CRΔ*zwf*, the wild-type) (Fig. 4A). However, on the NY containing 0.5% glucose tested plate, the *zwf* deletion mutant displayed severely weakened cell motility compared with the wild-type. The diameter of the growth zones resulting from migration away from the inoculation points was about 1.0 cm for the *zwf* deletion mutant, but 3.45 cm for the wild-type (Fig. 4B). As analyzed by the *t* test, the mean diameter of the *zwf* deletion mutant was remarkably shorter than that of the wild-type ($P = 0.01$) (Fig. 4B). There is no much difference in the diameter of the growth zones between the complemented strain (CRΔ*zwf*) and the wild-type ($P = 0.05$), which indicate that the cell motility of the *zwf* deletion mutant could be restored to the wild-type level by *zwf in trans*. These observations demonstrate that *zwf* of *Xoc* is implicated in cell motility.

The conversion of glucose 6-phosphate into gluconate 6-phosphate occurs mainly in ED and pentose phosphate pathways that play important roles in bacterial EPS production. This prompted us to determine whether deletion mutation in *zwf* has some effect on EPS production of *Xoc*. All *Xanthomonas* strains were grown in NB, NY medium alone and NY medium supplemented with 2% various carbohydrates for 5 days, respectively. EPS was extracted from the cultures (see Materials and Methods for details). As summarized in Fig. 5A, when grew in NY medium alone or NY medium containing 2% pyruvate, all of the strains which included in this experiment produced equal amount of EPS. However, the *zwf* deletion mutant produced about 15–45% less amount of EPS than the wild-type when cultured in NB, glucose-, fructose-, sucrose-, mannose- or galactose-containing NY medium. In addition, the EPS yield of the complemented mutant strain (CRΔ*zwf*) showed no significant difference from that of the wild-type. These results together suggest that *zwf* is involved in EPS production of *Xoc*.

3.5. zwf affects the transcript level of rpfF, rpfG and clp of Xoc

In order to investigate whether deletion mutation in *zwf* influences the transcript level of key genes in DSF-signaling network, a real-time quantitative PCR was performed to assess the transcript level of *rpfF*, *rpfG* and *clp*. The results show that the relative transcript level of *clp* significant decreased in *RΔzwf* than that in the wild-type. Intriguingly, the *rpfG* relative transcript level in *RΔzwf* evidently increased than that in the wild-type; and there was no

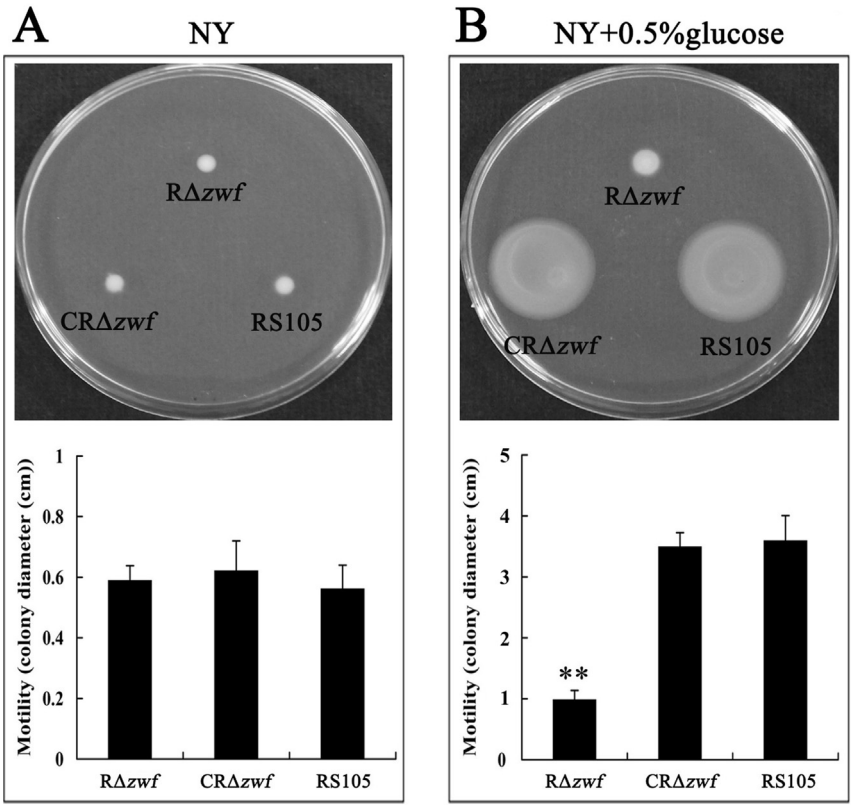

Fig. 4. Determination of cell motility of *Xoc* strains. (A) Cell motility of *RΔzwf* on NY medium plate. (B) Cell motility of *RΔzwf* on NY medium plates supplemented with 0.5% glucose. The fresh *Xanthomonas* bacteria cells adjusted to $OD_{600} = 0.3$ by sterilized distilled water were inoculated onto NY alone and NY supplemented 0.5% glucose plates containing 0.3% agarose. Photographs were taken 2 days after incubation at 28 °C. The following histogram is the measurement relative to the above photograph, respectively. Data are the mean ± SD of triplicate measurements from a representative experiment. The asterisks in each horizontal data column results from a paired, two-tailed Student *t* test relative to the wild-type. $^{**}P = 0.01$; $^{*}P = 0.05$.

significant difference in *rpfF* between two strains (*RΔzwf*, the wild-type) (Fig. 5B). These results suggested that *zwf*, involved in the conversion of glucose 6-phosphate into gluconate 6-phosphate, has effect on the transcript level of *rpfG* and *clp* in DSF-signaling network.

4. Discussion

In this study, we identified a novel *Xoc* virulence gene, *zwf*, which encodes glucose-6-phosphate dehydrogenase. *zwf*, which is highly conserved in *Xanthomonas* spp. and converts glucose 6-phosphate into gluconate 6-phosphate that implicates in the ED and pentose phosphate pathways. Here, we demonstrate that deletion mutation in *zwf* of *Xoc* impairs the growth capability of pathogen when hexoses (glucose, sucrose, fructose, mannose or galactose) was used as the sole carbon source, but does not affect its growth capability when pyruvate was used as the sole carbon (Fig. 2). It is important to note that the growth capability of the *zwf* deletion mutant was not impaired on NY medium (non-sugar), indicating that deletion mutation in *zwf* does not cause a growth defect. Our findings suggest that deletion mutation in *zwf* may hinder the ED and pentose phosphate pathways, but does not affect the gluconeogenesis pathway. This is consistent with previous reports that *Xanthomonas* spp. primarily employs ED, together with pentose phosphate pathway, to utilize hexoses [6,14,17,27,33]. It is worth noting that the growth capability of the *zwf* deletion mutant to utilize hexoses was not completely lost. The exact reason for this is still unknown. Probably, the downstream pathway of functional glycolysis or other compensatory pathways are operative in *Xoc*.

In *Xanthomonas* spp., EPS is an important virulence factor that plays a prominent role during pathogen infection [6,34]. It can enhance the susceptibility of host plant by suppressing defence responses such as callose formation [35], and make for biofilm formation [36] and prevent host recognition [37]. The biosynthesis of EPS in *Xoc* is a complex process that requires a large number of ATP and carbon precursors, just as in *Xcc* [14,38]. The *zwf* deletion mutant produces significantly less EPS compared with the wild-type when hexoses (glucose, sucrose, fructose, mannose or galactose) are used as the sole carbons (Fig. 5A). Probably, the deletion mutation in *zwf* of *Xoc* leads the impaired ability of pathogen to convert hexoses into gluconate 6-phosphate for ED and pentose phosphate pathways, and results in ATP diminution and short supply of carbon precursors for the synthesis of EPS. It could explain the reason why EPS production in the *zwf* deletion mutant is remarkably reduced. Recently, consistent or similar phenomena in *Xanthomonas* spp., are widely reported [6,14–18].

Cell motility allows bacteria to obtain more or better nutritional sources, avoid toxic substances or unfavorable environments, find a host, and disperse effectively [39,40]. It was also reported that cell motility makes contribution to invasion and colonization of pathogen, and the development of disease symptom [41,42]. Although the relationship between cell motility and virulence has not been specifically reported, cell motility and virulence factor EPS likely have certain cross-talk relations [43]. Thus, we speculate that cell motility more or less makes contribution to the bacterial full virulence. In *Xcc*, it has been demonstrated that RsmA, DsbB, PilZ and ADK, which are involved in cell motility, are required for the full virulence [42,43]. Deletion mutation in *zwf* of *Xoc* resulted in a

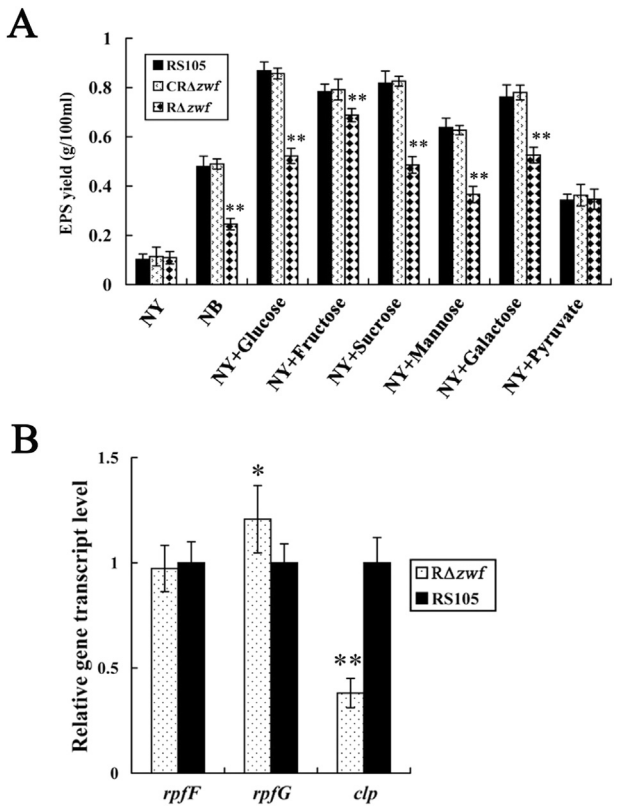

Fig. 5. (A) Determination of EPS products by *Xoc* strains. The fresh, equivalent *Xanthomonas* bacterial cells were cultured in NB, NY medium alone and NY medium supplemented with 2% various carbon sources (glucose, sucrose, fructose, mannose, galactose or pyruvate) with shaking at 200 rpm for 5 days at 28 °C, respectively. The bacterial cultures were collected for assay of EPS production. (B) Influence of deletion mutation in *zwf* on the transcript level of *rpfF*, *rpfG* and *clp* of *Xoc*. RNAs were isolated from cultures of the wild-type and *RΔzwf* grown in rice suspension cells for 16 h. The relative mRNAs transcript level of *rpfF*, *rpfG* and *clp* were calculated by real-time quantitative RT-PCR with respect to the level of the corresponding transcript in the wild-type, with the *16S rRNA* as the internal standard. Data are means ± SD from three replicates. The asterisks in each horizontal data column results from a paired, two-tailed Student *t* test relative to the wild-type. $^{**}P = 0.01$; $^{*}P = 0.05$.

significant reduction in cell motility compared with the wild-type (Fig. 4). Consistent with this phenomenon, we also found that *xanA* of *Xoc* plays an important role in cell motility and carbohydrate metabolism [44]. To our knowledge, ATP reduction probably is partially the reason for the weakened cell motility, which requires ATP to provide energy to drive activities.

It is well known that the carbohydrate metabolism genes could be induced by the presence of their substrates [6,30]. Thus, it promoted us to investigate the conditions under which the *zwf* promoter is activated. We found that *zwf* was induced in the presence of hexoses (glucose, sucrose, fructose, mannose or galactose) (Fig. 3). Furthermore, we also found that the transcript level of *zwf* is suppressed by malate. Probably, carbohydrate metabolism intermediates from the TCA cycle play an inhibitory role in it. Certainly, this hypothesis should be further verified. Deletion mutation in *zwf* of *Xoc* also resulted in the up-regulation of the transcript level of *rpfG*, but the down-regulation of the transcript level of *clp* (Fig. 5B). Similar phenomenon was also found in *Xoc*. The *pgi* deletion mutant not only altered the transcript level of *rpfG* and *clp*, but also impaired the ability of pathogen to produce DSF signal molecule (data not published). Hence, further works need to be done to prove whether the *zwf* deletion mutant also directly affected the production of DSF signal molecule. In short, the attenuation in virulence and growth in rice leaves of the *zwf* deletion mutant may result, at least partially if not completely, from the reduction in EPS synthesis and impaired ability to utilize hexoses (glucose, sucrose, fructose, mannose or galactose). Furthermore, *zwf* was also shown to be implicated in cell motility and the transcript level of key genes in DSF-signaling network, which perhaps also play a partial role in the full virulence of pathogen. Whether *zwf* has any other functions in carbohydrate metabolism or the full virulence is still under investigating.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.micpath.2014.11.007.

References

[1] Y.R. Li, H.S. Zou, Y.Z. Che, Y.P. Cui, W. Guo, et al., A novel regulatory role of HrpD6 in regulating *hrp-hrc-hpa* genes in *Xanthomonas oryzae* pv. *oryzicola*, Mol. Plant Microbe Interact. 24 (2011) 1086–1101.
[2] D.O. Niño-Liu, P.C. Ronald, A.J. Bogdanove, *Xanthomonas oryzae* pathovars: model pathogens of a model crop, Mol. Plant Pathol. 7 (2006) 303–324.
[3] W. Guo, Y.P. Cui, Y.R. Li, Y.Z. Che, L. Yuan, et al., Identification of seven *Xanthomonas oryzae* pv. *oryzicola* genes potentially involved in pathogenesis in rice, Microbiology 158 (2012) 505–518.
[4] L.F. Zou, X.P. Wang, Y. Xiang, B. Zhang, Y.R. Li, et al., Elucidation of the *hrp* clusters of *Xanthomonas oryzae* pv. *oryzicola* that control the hypersensitive response in nonhost tobacco and pathogenicity in susceptible host rice, Appl. Environ. Microbiol. 72 (2006) 6212–6224.
[5] Y.R. Li, Y.Z. Che, H.S. Zou, Y.P. Cui, W. Guo, et al., Hpa2 required by HrpF to translocate *Xanthomonas oryzae* transcriptional activator-like effectors into rice for pathogenicity, Appl. Environ. Microbiol. 77 (2011) 3809–3818.
[6] W. Guo, L.F. Zou, Y.R. Li, Y.P. Cui, Z.Y. Ji, et al., Fructose-bisphophate aldolase exhibits functional roles between carbon metabolism and the *hrp* system in rice pathogen *Xanthomonas oryzae* pv. *oryzicola*, PLoS One 7 (2012) e31855.
[7] W. Guo, L.L. Cai, H.S. Zou, X.L. Liu, L.F. Zou, et al., Ketoglutarate transport protein KgtP is secreted through the type III secretion system and contributes to virulence in *Xanthomonas oryzae* pv. *oryzae*, Appl. Environ. Microbiol. 78 (2012) 5672–5681.
[8] Y.W. He, L.H. Zhang, Quorum sensing and virulence regulation in *Xanthomonas campestris*, FEMS Microbiol. Rev. 32 (5) (2008) 842–857.
[9] Y.W. He, J. Wu, J.S. Cha, L.H. Zhang, Rice bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae* produces multiple DSF-family signals in regulation of virulence factor production, BMC Microbiol. 10 (2010) 187.
[10] B.M. Lee, Y.J. Park, D.S. Park, H.W. Kang, J.G. Kim, et al., The genome sequence of *Xanthomonas oryzae* pathovar *oryzae* KACC10331, the bacterial blight pathogen of rice, Nucleic Acids Res. 33 (2005) 577–586.
[11] H. Ochiai, Y. Inoue, M. Takeya, A. Sasaki, H. Kaku, Genome sequence of *Xanthomonas oryzae* pv. *oryzae* suggests contribution of large numbers of effector genes and insertion sequences to its race diversity, Jpn. Agric. Res. Q. 39 (2005) 275–287.
[12] S.L. Salzberg, D.D. Sommer, M.C. Schatz, A.M. Phillippy, P.D. Rabinowicz, et al., Genome sequence and rapid evolution of the rice pathogen *Xanthomonas oryzae* pv. *oryzae* PXO99^{A}, BMC Genomics 9 (2008) 204–221.
[13] A.J. Bogdanove, R. Koebnik, H. Lu, A. Furutani, S.V. Angiuoli, et al., Two new complete genome sequences offer insight into host and tissue specificity of plant pathogenic *Xanthomonas* spp, J. Bacteriol. 193 (19) (2011) 5450–5464.
[14] G.T. Lu, J.R. Xie, L. Chen, J.R. Hu, S.Q. An, et al., Glyceraldehyde-3-phosphate dehydrogenase of *Xanthomonas campestris* pv. *campestris* is required for extracellular polysaccharide production and full virulence, Microbiology 155 (2009) 1602–1612.
[15] C.H. Hsu, Y.M. Lo, Characterization of xanthan gum biosynthesis in a centrifugal, packed-bed reactor using metabolic flux analysis, Process Biochem. 38 (2003) 1617–1625.
[16] G.T. Lu, Z.J. Yang, F.Y. Peng, Y.N. Tan, Y.Q. Tang, et al., The role of glucose kinase in carbohydrate utilization and extracellular polysaccharide production in *Xanthomonas campestris* pathovar *campestris*, Microbiology 153 (2007) 4284–4294.

[17] S.Y. Kim, B.M. Lee, J.Y. Cho, Relationship between glucose catabolism and xanthan production in *Xanthomonas oryzae* pv. *oryzae*, Biotechnol. Lett. 32 (2010) 527–531.
[18] S. Blanvillain, D. Meyer, A. Boulanger, M. Lautier, C. Guynet, et al., Plant carbohydrate scavenging through TonB-dependent receptors: a feature shared by phytopathogenic and aquatic bacteria, PLoS One 2 (2007) e224.
[19] T.F. Watt, M. Vucur, B. Baumgarth, S.A. Watt, K. Niehaus, Low molecular weight plant extract induces metabolic changes and the secretion of extracellular enzymes, but has a negative effect on the expression of the type-III secretion system in *Xanthomonas campestris* pv. *campestris*, J. Biotechnol. 140 (2009) 59–67.
[20] D.J. Tang, Y.Q. He, J.X. Feng, B.R. He, B.L. Jiang, et al., *Xanthomonas campestris* pv. *campestris* possesses a single gluconeogenic pathway that is required for virulence, J. Bacteriol. 187 (2005) 6231–6237.
[21] J.H. Miller, Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1972.
[22] Y.L. Xiao, Y.R. Li, Z.Y. Liu, Y. Xiang, G.Y. Chen, Establishment of the *hrp*-inducing systems for the expression of the *hrp* genes of *Xanthomonas oryzae* pv. *oryzicola*, Acta Microbiol. Sin. 47 (2007) 396–401.
[23] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, NY, 1989. Cold Spring Harbor Laboratory.
[24] P.E. Turner, Phenotypic plasticity in bacterial plasmids, Genetics 167 (2004) 9–20.
[25] L.F. Zou, Y.R. Li, G.Y. Chen, A non-marker mutagenesis strategy to generate poly-*hrp* gene mutants in the rice pathogen *Xanthomonas oryzae* pv. *oryzicola*, Agric. Sci. China 10 (8) (2011) 1139–1150.
[26] J.L. Tang, Y.N. Liu, C.E. Barber, J.M. Dow, J.C. Wootton, et al., Genetic and molecular analysis of a cluster of *rpf* genes involved in positive regulation of synthesis of extracellular enzymes and polysaccharide in *Xanthomonas campestris* pathovar *campestris*, Mol. Gen. Genet. 226 (1991) 409–417.
[27] A.C. Zagallo, C.H. Wang, Comparative glucose catabolism of *xanthomonas* species, J. Bacteriol. 93 (1967) 970–975.
[28] R.G. Ankenbauer, E.W. Nester, Sugar-mediated induction of *Agrobacterium tumefaciens* virulence genes: structural specificity and activities of monosaccharides, J. Bacteriol. 172 (1990) 6442–6446.
[29] L.G. Rahme, M.N. Mindrinos, N.J. Panopoulos, Plant and environmental sensory signals control the expression of *hrp* genes in *Pseudomonas syringae* pv. *phaseolicola*, J. Bacteriol. 174 (1992) 3499–3507.
[30] D. Tamir-Ariel, T. Rosenberg, S. Burdman, The *Xanthomonas campestris* pv. *vesicatoria citH* gene is expressed early in the infection process of tomato and is positively regulated by the TctDE two-component regulatory system, Mol. Plant Pathol. 12 (2011) 57–71.
[33] F. Letisse, P. Chevallereau, J.L. Simon, N. Lindley, The influence of metabolic network structures and energy requirements on xanthan gum yields, J. Biotechnol. 99 (2002) 307–317.
[34] W. Yang, Y. Liu, L. Chen, T. Gao, B. Hu, et al., Zinc uptake regulator (*zur*) gene involved in zinc homeostasis and virulence of *Xanthomonas oryzae* pv. *oryzae* in rice, Curr. Microbiol. 54 (2007) 307–314.
[35] M.H. Yun, P.S. Torres, MEl Oirdi, L.A. Rigano, R. Gonzalez-Lamothe, et al., Xanthan induces plant susceptibility by suppressing callose deposition, Plant Physiol. 141 (2006) 178–187.
[36] J.M. Dow, L. Crossman, K. Findlay, Y.Q. He, J.X. Feng, et al., Biofilm dispersal in *Xanthomonas campestris* is controlled by cell-cell signaling and is required for full virulence to plants, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 10995–11000.
[37] A.M. Alvarez, Black rot of crucifers, in: A.J. Slusarenko, R.S.S. Fraser, L.C. van Loon (Eds.), Mechanisms of Resistance to Plant Diseases, Kluwer Academic Publications, Dordrecht, 2000, pp. 21–52.
[38] F. Letisse, P. Chevallereau, J.L. Simon, N.D. Lindley, Kinetic analysis of growth and xanthan gum production with *Xanthomonas campestris* on sucrose, using sequentially consumed nitrogen sources, Appl. Microbiol. Biotechnol. 55 (2001) 417–422.
[39] D. Tamir-Ariel, N. Navon, S. Burdman, Identification of genes in *Xanthomonas campestris* pv. *vesicatoria* induced during its interaction with tomato, J. Bacteriol. 189 (2007) 6359–6371.
[40] A. VandeBroek, J. Vanderleyden, The role of bacterial motility, chemotaxis, and attachment in bacteria-plant interactions, Mol. Plant Microbe Interact. 8 (1995) 800–810.
[41] J. Tans-Kersten, H. Huang, C. Allen, *Ralstonia solanacearum* needs motility for invasive virulence on tomato, J. Bacteriol. 183 (2001) 3597–3605.
[42] Y. McCarthy, R.P. Ryan, K. O'Donovan, Y.Q. He, B.L. Jiang, et al., The role of PilZ domain proteins in the virulence of *Xanthomonas campestris* pathovar *campestris*, Mol. Plant Pathol. 9 (2008) 819–824.
[43] G.T. Lu, Y.Q. Tang, C.Y. Li, R.F. Li, S.Q. An, et al., An adenosine kinase exists in *Xanthomonas campestris* pathovar *campestris* and is involved in extracellular polysaccharide production, cell motility, and virulence, J. Bacteriol. 191 (11) (2009) 3639–3648.
[44] W. Guo, C. Chu, X.X. Yang, Y. Fang, X. Liu, et al., Phosphohexose mutase of *Xanthomonas oryzae* pv. *oryzicola* is negatively regulated by HrpG and HrpX, and required for the full virulence in rice, Eur. J. Plant Pathol. 140 (2014) 353–364.
[45] J. Jiang, H.S. Zou, Y.R. Li, G.Y. Chen, Expression of the *hrcC, hrpE* and *hpa3* genes is not regulated by the *hrpG* and *hrpX* genes in a rice pathogen *Xanthomonas oryzae* pv. *oryzicola*, Wei Sheng Wu Xue Bao 49 (2009) 1018–1025.